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(54) Title: ENCAPSULATED PCR REAGENTS

(57) Abstract

Compositions and methods for hot start enzymatic reactions, particularly PCR, providing essential reactants encapsulated in a matrix which dissociates at a predetermined temperature.

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ENCAPSULATED PCR REAGENTS

FIELD OF THE INVENTION

This invention relates to reagent compositions for enzymatic reactions, and more particularly, to encapsulated reagents for active release at a specified reaction temperature. In a preferred embodiment, the invention includes enzymes or other required reagents encapsulated within a matrix and thereby isolated from a reaction mixture, which matrix dissociates at a desired temperature, releasing reactants to initiate a desired reaction.

BACKGROUND OF THE INVENTION

The polymerase chain reaction (PCR) is a chemical method for greatly increasing or amplifying the concentration of a specific nucleic acid sequence relative to that of other nucleic acids in the reaction mixture. A typical reaction includes the following reagents: (1) a target nucleic acid sequence; (2) at least two oligonucleotide primers complementary to the ends of the target sequence on opposite strands and oriented so that their 3'-OH ends point toward one another along the intervening sequence; (3) a polymerase enzyme; (4) mononucleotides; and (5) divalent metal ion (Mg^{2+}).

The PCR amplification reaction is generally carried out in a thermal cycler which provides repeating temperature cycles of (1) heating to cause strand separation (90-99°C); (2) cooling to permit primer-template annealing (30-60°C); and (3) warming to permit optimal polymerase activity and extension of the primers (70-75°C). The optimal temperature for

each phase in the thermocycling will vary with the characteristics of each specific target and set of primers. This cycle is repeated approximately 10-50 times to amplify the target nucleic acid sequence. The typical gain per cycle is slightly less than the theoretical maximum of 2.0X, such that operation through about 30 cycles can cause amplification approximately up to 5 X 10^8 times.

For a review of PCR Amplification Techniques, see for example Focus 16:38-57, 1994, and specifically, the "Tech-Line" notes on page 57, which is hereby incorporated by reference. For a general review of this technique, see, for example, Bloch, Biochemistry 30:2735-2747, 1991; and specific papers in the "PCR Bibliography" available from Perkin Elmer.

Many variations of the PCR technique have recently evolved, enhancing this powerful tool for use in nucleic acid sequencing, diagnostic testing, and therapeutics. However, inherent problems in the technique can cause problems of fidelity, resulting, for example, in amplification of nonspecific fragments that complicate or obscure results. The unwanted products are primarily due to nonspecific binding of primers under conditions of low stringency and low level activity of the thermostable polymerase during set-up and early portions of the reaction. Those reactions using complex genomic or cDNA templates, degenerate primers, very low copy number targets, large numbers of thermal cycles (e.g., greater than 35 cycles), or more than one target sequence in the same tube (multiplex PCR) are particularly prone to the generation of a variety of unwanted side reaction products. These nonspecific products can make the PCR results difficult to interpret and can reduce the yield of the desired specific fragment.

Hot Start PCR can improve the specificity and yield of PCR by isolating from the reaction an essential component such as the polymerase enzyme or primers until all other reaction components have been heated above the annealing temperature. The missing component is then added to each PCR reaction tube. Although hot start PCR can improve PCR performance, existing hot start methods are time-consuming, cumbersome, and prone to cross-contamination.

In one method, the PCR reaction tube containing all reactants except polymerase or primers is heated until a wax film is melted over the reaction components. See, for example, the 1994 Perkin Elmer technical bulletin entitled "AmpliWax™ PCR Gems." The tube is cooled to solidify the wax. The missing reagent is then pipetted atop the wax barrier, and the tubes returned to the thermocycler. The PCR temperature is then increased and the wax melts, allowing the missing reagents to mix with other reactants.

The wax barrier hot start is inconvenient due to the extra steps required in setting up the PCR reaction, e.g., melting the wax, cooling the wax, adding reagents and restarting the PCR reaction. Both manual and wax-barrier hot starts are associated with an increased risk of cross-contamination of samples because the tubes must be reopened before the amplification reaction is started. In addition, the wax begins to melt at about 50°C, permitting reactants to mix at temperatures well below typical annealing temperatures. Thus, this method does not solve the fidelity problems associated with PCR.

Alternative methods suggested to increase PCR specificity and yield include the addition of monoclonal antibodies to bind and inhibit the

polymerase enzyme during reagent assembly. (Kellogg et al., Clontechniques, 1X:1-4, 1994). The antibody-enzyme complex completely dissociates at about 60°C. While this method may improve performance, it adds additional cost and reagents to the reaction mixture. Further, at least some free enzyme exists in the reaction mixture at typical annealing temperatures. Thus, this method does not solve the problem of non-specificity, especially where the sample is in minute quantities and high background is present.

Another variation of the wax barrier technique isolates MgCl from the reaction mixture. The wax begins to melt at about 50°C permitting addition of Mg^{2+} to the reaction mixture. This method suffers the same drawbacks as conventional hot start PCR, because all reagents are present at typical annealing temperatures. In addition, trace amounts of Mg^{2+} are generally present in the reaction mixture and the polymerase retains some activity even in the absence of a divalent metal cation.

It would be highly desirable to provide a simple, efficient, and specific reagent composition and method for improving specificity and yield of PCR products.

SUMMARY OF THE INVENTION

It has now been found that reagents required for primer extension, including nucleic acid polymerase enzyme, can be stably immobilized within a hydrophilic matrix, which matrix is formed of a substance which dissociates at a specified temperature releasing active reagents. Specifically, polymerase enzyme and other PCR reagents can be encapsulated or entrapped in a matrix such as agarose which dissociates at a given temperature, releasing the reagents. In a preferred embodiment, nucleic acid polymerase, primers,

mononucleotides, Mg^{2+}, or mixtures of these reagents are combined with molten agarose and then cooled to form gelled agarose, e.g., beads, having entrapped reagents. Preferably, the reagent beads are coated with a barrier film such as paraffin wax to prevent desiccation of the agarose and to provide a condensation barrier, for example during PCR reactions.

The reaction beads are added, for example, to a PCR reaction tube containing a sample. Upon initiation of the thermal cycles, the reaction mixture is heated to reach the desired denaturation temperature. If present, a wax coating melts from the reaction bead as the tube is heated at approximately 50-55°C. The agarose matrix, however, does not dissociate to release its entrapped reagents until a predetermined temperature is reached, determined by the melting point of the matrix. In general, the matrix will dissociate between approximately 60°-95°C, depending upon the type and concentration of the matrix used. The encapsulated reagent composition thus provides a true hot start reaction for PCR or other enzymatic reactions.

In an alternative embodiment of this invention, the polymerase enzyme or other reagents are mixed with a melted hydrophilic matrix such as agarose and the mixture is coated onto a reaction surface such as the wells of a microtiter plate or walls of reactions tubes. The coated plates or reaction tubes may be used immediately or stored, preferably sealed from air or moisture for later use. Sample is added to the coated reaction surface, and the thermal cycle begun. In a similar manner as discussed for the reaction bead above, at the desired melting temperature, the matrix dissociates releasing its entrapped reagents for a true hot start reaction.

The encapsulated reagents of the present invention are generally packaged or coated to prevent desiccation and contamination, and are stable at room temperature for long periods of time. Compositions can be formulated with specific primers or reagents to provide a simple and reproducible method for one-step PCR reactions. Such a simple reaction reagent can be formulated as a kit containing encapsulated control and test reagents, initiating PCR with the addition of the encapsulated reagents to a test sample. Thus, the reagent composition of this invention can provide pre-aliquoted reagents for reproducible, error-free, and true hot start PCR. Multiple analysis can be accomplished, for example on a microtiter test plate, with specified wells containing varied control and test reagents.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a photograph of an ethidium bromide-stained agarose gel showing the MSP gene of *C. elegans* amplified by the reactions described in Example 1.

Lane 1: control, agarose bead, no wax, oil atop;

Lane 2: control, wax-coated agarose bead;

Lane 3: Test-1, wax-coated agarose-encapsulated *Taq* DNA polymerase;

Lane 4: Test-2, wax-coated agarose-encapsulated *Taq* DNA polymerase and primers.

Figure 2 is a photograph of an ethidium bromide-stained agarose gel showing the MSP gene of *C. elegans* amplified in the presence and absence of human blood (lanes 1 and 2) and the human beta-globin gene amplified from a human blood sample (lanes 3-5) by the reactions described in Example 2.

Lane 1: Control-1, MSP gene, no agarose, no blood;
Lane 2: Control-2, MSP gene, no agarose, blood;
Lane 3: Control-3, hβG gene, no agarose, oil atop;
Lane 4: Test-1, hβG gene, wax-coated agarose-encapsulated *Taq* DNA polymerase;
Lane 5: Test-2, hβG gene, wax-coated, agarose-encapsulated *Taq* DNA polymerase and primers.

Figure 3 is a photograph of an ethidium bromide-stained agarose gel showing MSP gene of *C. elegans* amplified by the reactions described in Example 4.
Lane 1: control, agarose coated onto reaction tube;
Lane 2: test, agarose-encapsulated *Taq* DNA polymerase coated onto reaction tube.

Figure 4 is a photograph of an ethidium bromide-stained agarose gel showing the MSP gene of *C. elegans* amplified by the reactions described in Example 5.
Lane 1: control, *Taq* DNA polymerase kept at room temperature 30 days prior to PCR;
Lane 2: test, agarose-encapsulated *Taq* DNA polymerase kept at room temperature 30 days prior to PCR.

Figure 5 is a photograph of an ethidium bromide-stained agarose gel showing the MSP gene of *C. elegans* amplified by the reactions described in Example 6.
Lane 1: Low melting point (LMP) agarose-encapsulated polymerase (5.0 units), high copy number (10^4);
Lane 2: high melting point (HMP) agarose-encapsulated polymerase (5.0 units), high copy number (10^4);

Lane 3: HMP agarose-encapsulated polymerase (2.5 units), high copy number (10^4);

Lane 4: LMP agarose-encapsulated polymerase (5.0 units), low copy number (10^3);

Lane 5: HMP agarose-encapsulated polymerase (5.0 units); low copy number (10^3);

Lane 6: HMP agarose-encapsulated polymerase (2.5 units), low copy number (10^3).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In its simplest form, the present invention provides a composition for use in chemical reactions which includes reactants encapsulated in a matrix formulated to dissociate and release its contents at a desired reaction temperature. Upon addition of sample and heating to the appropriate reaction temperature, the matrix dissociates, releasing a premeasured amount of reactants into the reaction mixture. The reaction units may be formed as beads or coated onto a reaction surface such as the inner surface of a reaction tube or microtitre well.

In a preferred embodiment useful in hot start reactions, reactants are encapsulated in a matrix which dissociates at a desired hot reaction temperature. For example, it is highly desirable to isolate certain specific PCR reactants such as polymerase enzyme, oligonucleotide primers, mononucleotides, Mg^{++}, or combinations thereof from the reaction mixture until the reaction temperature has passed annealing temperature, e.g., approximately T_m, which generally is greater than approximately 60°C.

Annealing temperature is the temperature above which the oligonucleotide primers and the DNA template melt or dissociate. The annealing temperature will

vary with the sequence homology between the template and the primers and the length and GC content of the primer and template. The annealing temperature at which 50% of a primer is annealed to its template (T_m) as well as an optimal annealing temperature for a specific reaction can be calculated, for example as described in Rychlik et al., Nucleic Acids Research, 18:6401-6412, 1990. In general, most PCR reactions utilize annealing temperatures in the range of from about 50°C to about 75°C.

The specificity or fidelity of PCR primer-template annealing is a function of temperature. That is, the higher the temperature, the less likely mispriming will occur. It is therefore desirable in many situations to conduct PCR at high stringency (high temperatures), e.g., greater than 60°C. Prior to the present invention, no hot start technique save manual reagent addition could exceed typical annealing temperatures, and all are therefore prone to mispriming and subsequent spurious PCR product formation.

Hot start PCR reactions have been achieved by isolating polymerase, primers, mononucleotides, or magnesium from other reactants with a barrier film of paraffin wax. The wax melts at approximately 50-55°C, permitting mixing of the previously isolated reactants with the reaction mixture. While this method may improve the quality of the PCR reaction, it does not achieve a true hot start PCR reaction above annealing temperatures and still suffers from some non-specific priming.

By incorporating the polymerase enzyme, e.g., DNA polymerase such as *Taq* DNA Polymerase and/or other reactants into a matrix such as agarose, the release of the reactants at a desired hot start temperature is achieved. The matrix is preferably hydrophilic, and

preferably dissociates at a reaction temperature greater than approximately 60°C. The most preferred dissociation temperature range is from about 64°C to about 95°C. For high stringency reactions, a preferred dissociation temperature is about 90°C.

Low melting point agarose is commercially available, and melts or dissociates at approximately greater than 60°C. High melting point agarose dissociates at approximately greater than 90°C. Other forms of agarose are commercially available which exhibit melting points between 60°C and 95°C. See, for example, the 1992 Sigma Chemical Co. catalog and the 1994 FMC Bio Products Catalog, which show types of agarose having varied melting temperatures which may be purchased. By varying the type and/or concentration of a particular type of agarose, a variety of specific remelting temperatures can be achieved, e.g., compositions having dissociation temperatures between approximately 60°C and 95°C can be formulated.

To achieve a desired remelting temperature of an agarose composition, a type of agarose have a melting point in the generally desired remelting temperature range is selected. For example, if the desired remelting temperature is approximately 92°C, high melting point agarose is selected (MP ≈ 94°C). Agarose solutions of various concentrations (e.g. 0.1-1.0%) are prepared in aqueous solution, e.g., water, heated to melting and cooled to gelling point in a reaction tube. The agarose reaction tubes are then reheated and the remelting points observed. In this way, an appropriate agarose and its concentration, for example that which remelted at 92°C can be selected. This selection process may be utilized to select a specific matrix type and concentration to achieve a desired reaction composition of the present invention.

The present invention permits encapsulation and isolation of active reagents from a reaction mixture until a desired temperature is reached. This is particularly useful in nucleic acid polymerization or extension methods, including primer extension methods which are prone to spurious results because of low temperature mispriming. The term "nucleic acid polymerization" includes any reaction in which a nucleic acid sequence is extended by the addition of mononucleotides. Nucleic acid polymerization includes, for example, PCR, nucleic acid sequencing, reverse transcriptase polymerization and nick translation. See, for example, Focus 16:38-57 (1994).

In its simplest form, the composition of the present invention includes one or more reagents required to initiate nucleic acid polymerization, the reagent(s) encapsulated within a matrix which dissociates at a predetermined temperature. Preferably, the encapsulated reagents include one or more of nucleic acid polymerase, primers, mononucleotides, and divalent metal cations. Most preferably, the composition includes nucleic acid polymerase and/or primers.

PCR reagents including polymerase, primers, mononucleotides, and divalent metal cations, remain stable at room temperature when encapsulated within an agarose matrix. The storage time of the encapsulated reagents may be extended by coating the surface of the matrix with a barrier to avoid desiccation of the matrix, e.g., with a thin coating of paraffin or by storing in an evacuated or sealed container. For example, DNA polymerase encapsulated in agarose beads and dipped in paraffin remains stable at room temperature for at least several months.

Variations in the above-described invention will be obvious to those of skill in the art. For example, the encapsulating matrix may be formed of a variety of materials, polymers, and the like, provided the matrix dissociates at the desired reaction temperature and is compatible with the desired reaction, that is, does not interfere with the reaction sequence or its products. To test for compatibility of a given matrix with the desired reaction, e.g., PCR, the reaction is carried out in the presence of the matrix over a range of matrix concentrations. The reaction is monitored for interference as compared with a matrix-free control.

Any number of reagents may be encapsulated within the matrix, providing a unique method of quality control and reproducability of reaction conditions. Reagent contamination, inactivation and waste are greatly reduced by the single-reaction unit provided by the instant composition. For example, in the preferred embodiment for hot start PCR, the matrix may include polymerase and/or primers, and optionally all other necessary reactants such as buffers, salts, mononucleotides, and the like. In this way, a user simply adds the encapsulated reagent unit to a reaction tube containing sample. No further additions are required.

To prepare the composition of the present invention, the one or more reactants are mixed with molten matrix, and the mixture is permitted to gel, encapsulating the reagents. The reactants may include solid, solvated, or aqueous solutions or dispersions of the compounds. Preferably, aqueous solutions of the reactants are mixed with the molten matrix, e.g., molten agarose.

In the present invention, various types of reagents are encapsulated in a matrix which dissociates

at a given desired reaction temperature to release active reagents. In a preferred embodiment, the encapsulated reagents include enzymes, such as polymerase enzyme for PCR reactions. Polymerase enzymes are commercially available, such as *Taq* DNA polymerase, *Vent* DNA polymerase, *Tth* DNA polymerase, and the like (New England Biolabs). In general, a 100μl PCR uses approximately 1-5 units of polymerase, typically available in about 1-2.5μl of the commercial enzyme. In the present invention, enzyme is added to molten matrix to yield a desired final reaction concentration. For example, an amount of *Taq* DNA polymerase (1-10 units) can be added to 5-9μl molten agarose and formed into a 10μl bead. Using agarose-*Taq* polymerase compositions, successful PCR with as little as 1.25 units of polymerase, even at low template copy number has been achieved.

Oligonucleotide primers may also be encapsulated in the matrix. Generally, the amount of primer added to matrix is sufficient to yield approximately 0.1-1μM of each primer in the final reaction volume. Primers are typically at least 15 base pairs in length, and generally range from 30-60 base pairs. Primers may also include restriction enzyme sites and may be labeled, e.g., radio, fluorescent or biotin-labeled.

Mononucleotides are essential to the elongation of primers in PCR and other polymerization methods. Monomers may be encapsulated in the matrix, generally in a concentration range of about 50 to about 200 μM for each monomer. Useful monomers are deoxyadensosine, guanosine, cytosine and thymine triphosphates, (dNTPs) and analogs thereof. Monomers may be labeled or modified as known to those of skill in the art.

The encapsulated reagent unit may be adapted by one of skill in the art for particular reactions. For

example, to facilitate multiplex PCR applications, a multi-stage unit can be used. An initial matrix, e.g., of high melting point agarose (>90°C) is used to encapsulate a first specific primer, and/or other reactants and is formed into a bead. This bead is then coated with a second matrix, e.g., of low melting point agarose (>60°C) encapsulating a different (second) set of primers, and optionally DNA polymerase and other PCR reactants. The multi-layered bead is preferably coated with paraffin wax. In its use in the PCR reaction, the multi-layered bead is first run through approximately 10 "cold" cycles with a maximum temperature of less than 90°C causing reaction with the second set of primers. Continuing cycles are then run at approximately >94°C to invoke reaction with the first specific set of primers. Variations in these techniques will be readily apparent to those of skill in the art.

The following examples further illustrate the invention.

EXAMPLE 1

PCR using agarose-encapsulated polymerase

Agarose beads incorporating PCR reactants were formed by melting 0.8% low melting point agarose (Sigma Chemical Co., St. Louis, MO) and equilibrating to approximately 60°C. One microliter (5 units) of *Taq* DNA Polymerase (Promega, Madison, WI) was mixed with 9 microliters of melted agarose. One microliter of *Taq* polymerase, two microliters of a 20 μM stock of primer p38 and two microliters of a 20 μM stock of primer p39 were added to five microliters of melted agarose. The sequences of these primers are listed below:

Primer Number	Sequence ID No.	Sequence
p38	1	5'-GGTTCAGAATTCATATGGCCCAATCCGTCCCAC-3'
p39	2	5'-GCAAGCTTAGATCTATGGGTTGTACTCAATTGG-3'

Control agarose without any entrapped reagents was also used. The agarose solutions were dropped onto an ice cooled parafilm sheet to form beads. The beads were then dipped 3 times into melted paraffin wax.

PCR was carried out using as sample template the plasmid pCEO1. pCEO1 was constructed by digesting the plasmid p3L4 (Ward et al., Journal of Molecular Biology, 199:1-13, 1988) with Hind III and Bgl III to release a 591 base pair fragment containing the MSP-56 gene of *C. elegans*. The DNA fragment was then ligated into the Hind III-Bam HI site of pUC18 (Yanisch-Perron et al., Gene 33:103-119, 1985). Primers p38 and p39 specifically amplify the major sperm protein gene (MSP) of *C. elegans*, approximately 380 base pairs in length. The PCR reaction tubes were set-up as follows:

REAGENT	CONTROL-1 40 µl mineral oil atop mixture	CONTROL-2 Wax-coated agarose bead	TEST-1 Encapsulated Polymerase	TEST-2 Encapsulated Polymerase and Primers
pCEO1 sample (0.4-0.5mg/ml)	1 µl	1 µl	1 µl	1 µl
10X sample buffer	10 µl	10 µl	10 µl	10 µl
$MgCl_2$ (25 mM)	8 µl	8 µl	8 µl	8 µl
dNTPs (10 mM)	8 µl	8 µl	8 µl	8 µl
p38 primer (20µM)	2 µl	2 µl	2 µl	[2 µl]
p39 primer (20µM)	2 µl	2 µl	2 µl	[2 µl]
Taq polymerase (5U/µl)	1 µl	1 µl	[1 µl]	[1 µl]
dH_2O	58 µl	58 µl	59 µl	58 µl
agarose bead	10 µl	-	-	-
agarose bead-wax	-	10 µl	10 µl	10 µl
Total Volume	100 µl	100 µl	100 µl	100 µl
Gel Lane	1	2	3	4
[] brackets indicate reagents encapsulated within agarose bead				

The first control reaction was run according to traditional PCR procedure, with mineral oil placed atop the reaction mixture to avoid condensation. In the second control reaction, a wax-coated agarose bead containing no PCR reactants was added to the reaction mixture. In the test samples, agarose beads having incorporated polymerase or polymerase and primers as discussed above were used.

The reaction tubes were capped and placed in a Perkin Elmer Cetus DNA Thermal Cycler Model No. 480 (Perkin Elmer, Norwalk, CT). The PCR conditions included approximately three minutes heating to reach 94°C, followed by repeating cycles of one minute at

94°C; thirty seconds at 58°C; and thirty seconds at 72°C for thirty cycles.

After PCR was completed, eight microliters of each reaction product was mixed with two microliters of bromphenol blue dye and applied to a 0.8% agarose gel stained with ethidium bromide to separate and analyze the DNA reaction product. As shown in Figure 1, all lanes show the target, 380 base pair MSP band.

EXAMPLE 2

Amplification of beta-globin from human blood

Whole blood was collected into a sample tube after pricking a human finger with a sterile lancet. One microliter of whole blood was used in each reaction mixture as sample from which beta-globin was to be amplified. The primers P1 and P2 as described in Bauer, et al., JAMA 265:472-477, 1992 were used to amplify the 293 base pair human beta-globin gene. The primer sequences were:

Primer Number	Sequence ID No.	Sequence
p1	3	5'-CAACTTCATCCACGTTCACC-3'
p2	4	5'-GAAGAGCCAAGGACAGGTAC-3'

As a control, the MSP gene was amplified from *C. elegans* DNA as described for Example 1 in the presence or absence of 1 µl human blood. The reaction mixtures were set up as follows:

REAGENT	CONTROL-1 No Agarose, 40µl Oil	CONTROL-2 No Agarose, 40µl Oil	CONTROL-3 No Agarose 40µl Oil	TEST-1 Encapsulated Polymerase	TEST-2 Encapsulated Polymerase and Primers
blood	-	1 µl	1 µl	1 µl	1 µl
pCEO1	1 µl	1 µl	--	--	--
10X buffer	10 µl	10 µl	10 µl	10 µl	10 µl
$MgCl_2$ (25 mM)	8 µl	8 µl	8 µl	8 µl	8 µl
dNTPs (10 mM)	.8 µl	8 µl	8 µl	8 µl	8 µl
H_2O	68 µl	67 µl	67 µl	59 µl	63 µl
primer* (20µM)	p38 2 µl	p38 2 µl	p1 2 µl	p1 2 µl	p1 [2 µl]
primer* (20µM)	p39 2 µl	p39 2 µl	p2 2 µl	p2 2 µl	p2 [2 µl]
polymerase (2 U/µl)	1 µl	1 µl	1 µl	[1 µl]	[1 µl]
agarose bead-wax	-	-	-	10 µl	10 µl
Total Volume	100	100	100 µl	100 µl	100 µl
Gel Lane	1	2	3	4	5

Gel Lane	1	2	3	4	5
*Primers p1 and p2 amplify the 293 base pair human β-globin gene. Primers p38 p39 amplify the 380 base pair *C. elegans* MSP gene.					
[] brackets indicate reagents encapsulated within agarose bead					

The reaction tubes were placed in a thermal cycler and PCR initiated and carried through 30 cycles as described for Example 1. The results of the PCR are shown in Figure 2 and demonstrate improved reaction product when the human beta-globin gene is amplified from a blood sample by the hot start agarose bead methods over conventional PCR. In lane 3, the control, conventional PCR shows very little production of the

desired 293 base pair product and the presence of unwanted bands. In contrast, both lanes showing reaction product produced from the inventive agarose bead hot start reactions clearly showed the desired 293 base pair product with little or no unwanted bands.

EXAMPLE 3

Agarose beads with varied melting points

Agarose beads formed of varying concentrations of high melting point agarose were evaluated for the temperature at which they dissociated and released their encapsulated contents. Solutions of 0.25%, 0.5% and 1.0% high melting point agarose were prepared, melted, and mixed with bromophenol blue dye, and formed into 10 µl beads as described above. The beads were dipped three times in paraffin and placed in 100µ of water. The tubes were placed in a thermocycler and slowly heated to controlled temperatures. The dissociation temperature was identified by release of dye from the bead into the surrounding solution.

% HMP AGAROSE	DISSOCIATION TEMPERATURE
0.25%	85°C
0.50%	92°C
1.0 %	> 94°C

EXAMPLE 4

Agarose encapsulated PCR reagents coated onto walls of reaction tubes

Low melting point agarose (0.8%) was prepared as described for Example 1 with and without *Taq* DNA Polymerase. The molten agarose-reagent solution was coated onto the reaction surface of 500 µl reaction tubes by dripping 10 µl of the agarose-reagent solution into a tube and swirling to coat the bottom and the lower portion of the side walls of the tube. The tubes

were cooled at room temperature for approximately 10 minutes to allow the agarose to gel. The reaction tubes were set-up by adding the appropriate reagents to the test tubes, and layering approximately 40 µl mineral oil atop each mixture.

REAGENT	CONTROL AGAROSE	TEST Encapsulated Polymerase
*pCEO1 sample (0.4-0.5 mg/ml)	1 µl	1 µl
10X sample buffer	10 µl	10 µl
$MgCl_2$ (25 mM)	8 µl	8 µl
dNTPs (10 mM)	8 µl	8 µl
p38 primer (20 µM)	2 µl	2 µl
p39 primer (20 µM)	2 µl	2 µl
Taq polymerase (20 U/µl)	1 µl	[1 µl]
dH_2O	58 µl	59 µl
agarose coat	10 µl	10 µl
Total volume	100 µl	100 µl
Gel Lane	1	2
[] brackets indicate reagents within agarose		

PCR was carried out and results analyzed as described for Example 1. As shown in Figure 3, both control and test lanes show the target, 380 base pair MSP band. Incorporation and immobilization of PCR reagents onto a reaction surface provided an efficient hot start PCR product.

EXAMPLE 5

STABILITY OF AGAROSE-ENCAPSULATED POLYMERASE

Agarose beads containing *Taq* Polymerase were prepared as described for Example 1. Agarose-encapsulated polymerase beads, as well as capped tubes containing *Taq* DNA Polymerase were permitted to remain at room temperature. After 30 days, PCR reactions was performed comparing activity of encapsulated *Taq* Polymerase with non-encapsulated polymerase. PCR was performed as described for Example 1, the only difference between the samples being the presence of *Taq* DNA polymerase within or outside the agarose bead. Results are shown in Figure 4, and demonstrate greater product yield in the reaction having encapsulated polymerase (lane 2) than the reaction using non-encapsulated polymerase. Further, the data indicate the encapsulated polymerase may be stored at room temperature for long periods of time without loss of activity.

EXAMPLE 6

COMPARISON OF HOT START TEMPERATURES

Agarose beads containing *Taq* DNA polymerase were prepared as described for Example 1 either low melting point or high melting point agarose. The remelting temperatures for these agarose preparations were determined to be 64°C and 92°C, respectively, by the method described for Example 3. The higher-melting agarose beads contained either 2.5 units or 5.0 units of *Taq* DNA Polymerase, the lower-melting point agarose beads contained 2.5 units.

PCR was carried out as described for Example 1 using either high copy number (10^4) target or low copy number (10^3) target, through an initial three minutes at 94°C, followed by 35 cycles of one minute at 94°C,

30 seconds at 50°C; and 30 seconds at 72°C. The amplified product was analyzed as described for Example 1. The results, shown in Figure 5, show amplification of the desired 380 bp product in all samples including high copy number (lanes 1-3) and low copy number (lanes 4-6). At high copy number, little difference was seen between low melting point agarose (64°C, lane 1) and high melting point agarose (92°C, lanes 3 and 4), but at low copy number the fidelity of the product was compromised when low melting point agarose (64°C, lane 4) was used as compared with high melting point agarose (92°C, lanes 5 and 6).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Smith, G. Kenneth

(ii) TITLE OF INVENTION: ENCAPSULATED PCR REAGENTS

(iii) NUMBER OF SEQUENCES: 4

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(C) CITY: Houston
(D) STATE: Texas
(E) COUNTRY: USA
(F) ZIP: 77027

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kettelberger, Denise M.
(C) REFERENCE/DOCKET NUMBER: 67347/1

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGTTCAGAAT TCATATGGCC CAATCCGTCC CAC

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCAAGCTTAG ATCTATGGGT TGTACTCAAT TGG

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAACTTCATC CACGTTCACC

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAAGAGCCAA GGACAGGTAC

CLAIMS

I claim:

1. A composition comprising:

a matrix which dissociates at a predetermined temperature, said temperature in the range of from about 60°C to about 95°C; and

one or more reagents required for nucleic acid polymerization encapsulated within said matrix.

2. The composition of claim 1, wherein the matrix dissociates at a temperature in the range from about 64°C to about 95°C.

3. The composition of claim 1, wherein the matrix dissociates at a temperature of about 90°C.

4. The composition of claim 1, wherein the matrix is formed of agarose.

5. The composition of claim 1, wherein said one or more reagents is selected from the group consisting of:

nucleic acid polymerase, mononucleotides, Mg^{++}, nucleic acid primers, and mixtures thereof.

6. The composition of claim 5, wherein said one or more reagents is DNA polymerase.

7. The composition of claim 1 further comprising a hydrophobic barrier film coating the reagent-encapsulated matrix.

8. The composition of claim 7, wherein said barrier film is wax.

9. The composition of claim 1, formed as multiple layers of reagent-encapsulated matrix, wherein each layer comprises a matrix having a different dissociation temperature.

10. A method for initiating nucleic acid polymerization at a selected reaction temperature comprising the steps of:

adding to a test sample a composition comprising one or more reagents required for nucleic acid polymerization, said one or more reagents encapsulated in a matrix which dissociates at a predetermined temperature;

heating the sample-reagent mixture to at least the predetermined temperature to dissociate and release the contents of the matrix, thereby initiating nucleic acid polymerization.

11. The method of claim 10, wherein said predetermined temperature is in the range of from about 60°C to about 95°C.

12. The method of claim 10 wherein said composition comprises said one or more reagents encapsulated in agarose.

13. The method of claim 10 wherein said one or more reagents is selected from the group consisting of nucleic acid polymerase, mononucleotides, Mg^{++}, nucleic acid primers, and mixtures thereof.

14. The method of claim 13 wherein said one or more reagents is DNA polymerase.

15. A reaction vessel having a surface which is at least partially coated with the composition of claim 1.

16. The reaction vessel of claim 15, wherein the vessel is selected from the group consisting of:
microtiter test well;
test tube; and
microscope slide.

17. The reaction vessel of claim 15 wherein said composition comprises said one or more reagents encapsulated in agarose.

18. The reaction vessel of claim 15, wherein said one or more reagents is selected from the group consisting of:
nucleic acid polymerase, mononucleotides, Mg^{++}, nucleic acid primers, and mixtures thereof.

19. The reaction vessel of claim 18, wherein said one or more reagents is DNA polymerase.

20. A composition comprising:
a premeasured unit dose of one or more reagents required for nucleic acid polymerization encapsulated within a matrix which dissociates at a predetermined temperature in the range of from about 60°C to about 95°C.

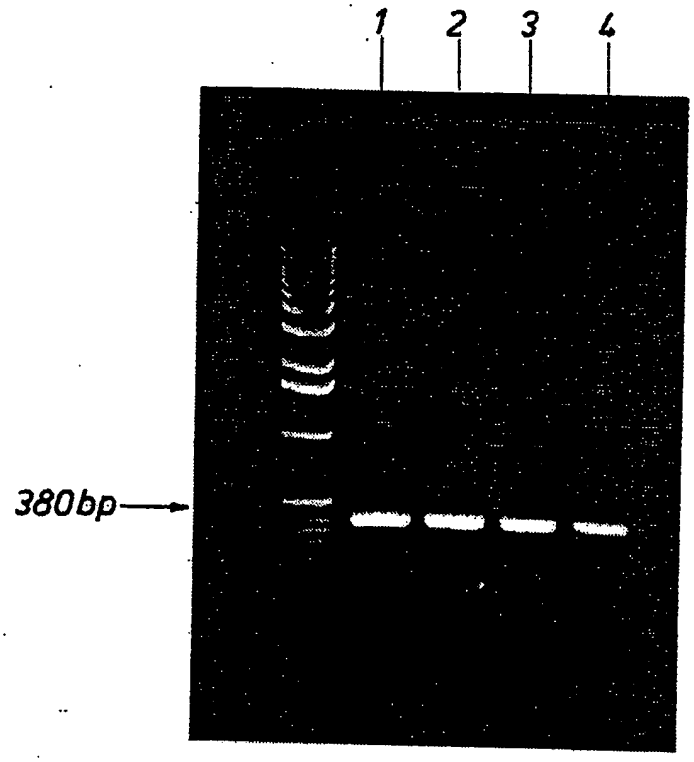


FIG. 1

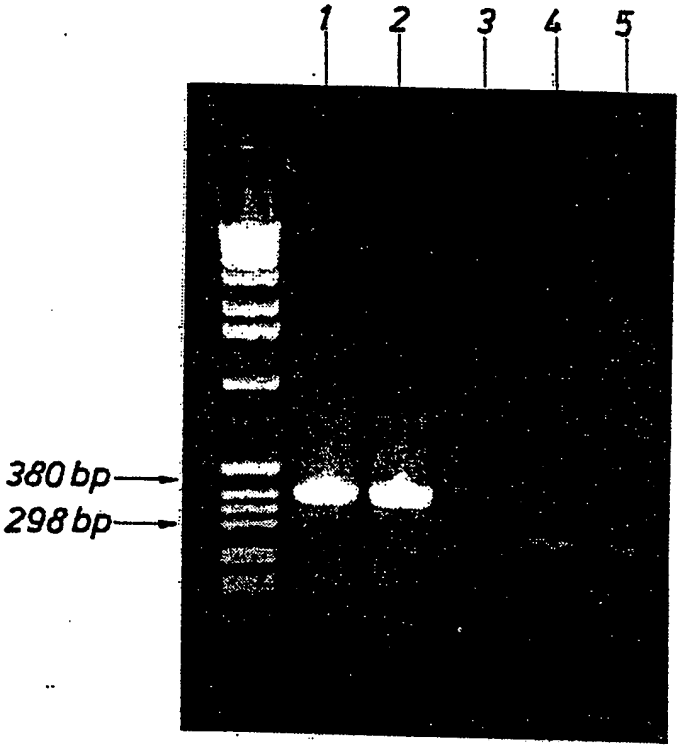


FIG. 2

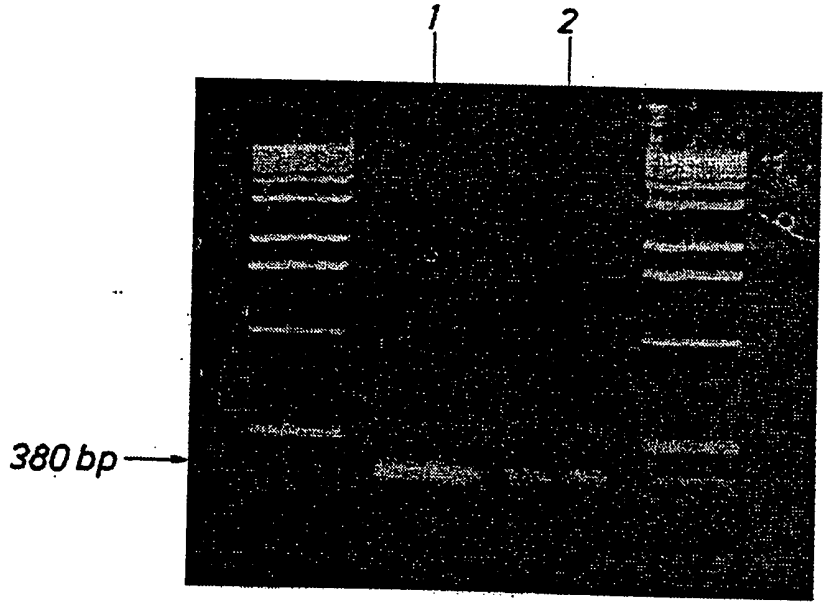


FIG. 3

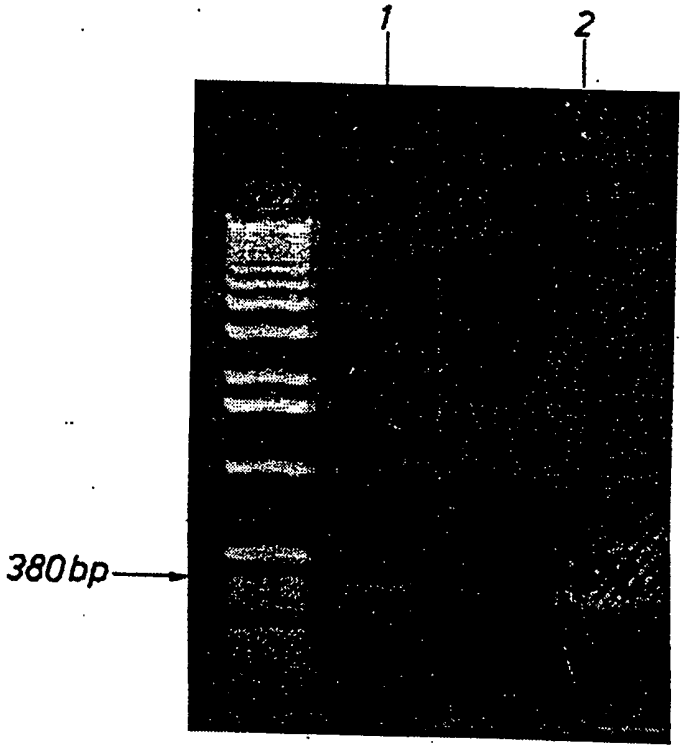
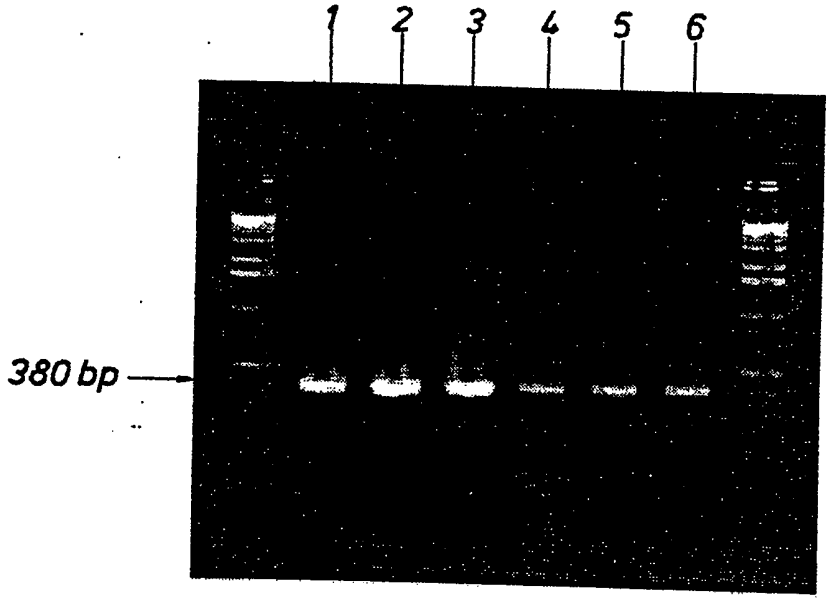


FIG. 4

FIG. 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07959

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34
US CL :435/6, 91.1, 91.2; 206/219, 568, 569
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.2; 206/219, 568, 569

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS
search terms: nucleic acid, polymerization, amplification, PCR, wax, agarose, encapsulate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO, A, 91/12342 (CETUS CORPORATION) 22 August 1991, see entire document.	1-3, 5-8, 10-11, 13-16, 18-20 --- 9
X -- Y	EP, A, 0,572,057 (EASTMAN KODAK COMP.) 01 December 1993, see entire document.	1-3, 5-8, 10-11, 13-16, 18-20 --- 9
Y	US, A, 5,053,332 (COOK ET AL) 01 October 1991, see entire document.	4, 17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 AUGUST 1995	06 OCT 1995
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